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The specific transmission of *Grapevine fanleaf virus* by its nematode vector *Xiphinema index* is solely determined by the viral coat protein

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Abstract

The viral determinants involved in the specific transmission of *Grapevine fanleaf virus* (GFLV) by its nematode vector *Xiphinema index* are located within the 513 C-terminal residues of the RNA2-encoded polyprotein, that is, the 9 C-terminal amino acids of the movement protein ($2B^{MP}$) and contiguous 504 amino acids of the coat protein ($2C^{CP}$) [Virology 291 (2001) 161]. To further delineate the viral determinants responsible for the specific spread, the four amino acids that are different within the 9 C-terminal $2B^{MP}$ residues between GFLV and *Arabis mosaic virus* (ArMV), another nepovirus which is transmitted by *Xiphinema diversicaudatum* but not by *X. index*, were subjected to mutational analysis. Of the recombinant viruses derived from transcripts of GFLV RNA1 and RNA2 mutants that systemically infected herbaceous host plants, all with the $2C^{CP}$ of GFLV were transmitted by *X. index* unlike none with the $2C^{CP}$ of ArMV, regardless of the mutations within the $2B^{MP}$ C-terminus. These results demonstrate that the coat protein is the sole viral determinant for the specific spread of GFLV by *X. index*.

Keywords: *Grapevine fanleaf virus*; *Xiphinema index*; Transmission; Virus-vector specificity; Coat protein; *Arabis mosaic virus*; Substitution mutations

Introduction

Grapevine fanleaf virus (GFLV) is responsible for a progressive degeneration of grapevines that occurs in most vineyards worldwide (Martelli and Savino, 1991). It causes serious economic losses by substantially reducing yield by up to 80% and affecting fruit quality.

GFLV belongs to the genus *Nepovirus* in the family *Comoviridae* (Mayo and Robinson, 1996). Particles are isometric and about 30 nm in diameter. The viral genome is composed of two single-stranded positive-sense RNAs, denoted RNA1 and RNA2, which carry a small covalently linked viral protein (VPg) at their 5′ extremities and a poly(A) stretch at their 3′ ends (Pinck et al., 1988) (Fig. 1). Each genomic RNA codes for a polyprotein that is proteolytically processed into functional proteins. RNA1 codes for the proteins implicated in RNA replication (Ritzenthaler et al., 1991) and for the viral proteinase (Margis et al., 1991) (Fig. 1). RNA2 codes for a protein ($2A^{HP}$) which is required for RNA2 replication and could act as homing protein (Gaire et al., 1999), the movement protein ($2B^{MP}$) which is the constituent protein of tubules observed in plasmodesmata (Ritzenthaler et al., 1995), and the coat protein ($2C^{CP}$) (Serghini et al., 1990) (Fig. 1). Based on strong similarities in virus spread between GFLV and *Cowpea mosaic virus* (CPMV), the type member of the family *Comoviridae*, functional proteins $2B^{MP}$ and $2C^{CP}$ are likely required for systemic plant infection through cell-to-cell and long distance movement (Belin et al., 1999; Carvalho et al., 2003). Both genomic RNAs are needed for infectivity in planta and full-length cDNA clones of GFLV RNA1 and RNA2 have been constructed for the synthesis of infectious transcripts (Viry et al., 1993).

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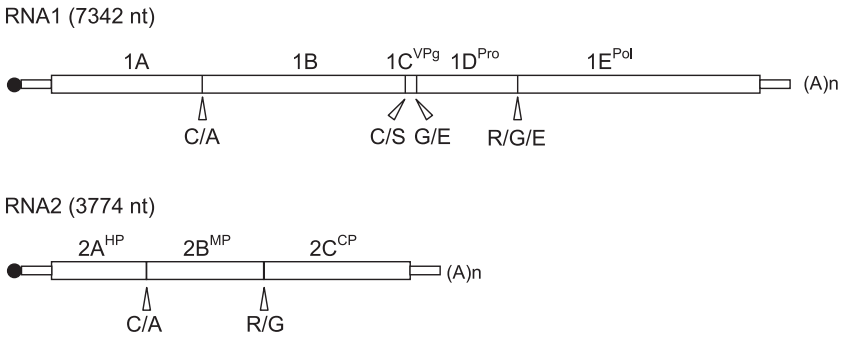

Fig. 1. Genetic organization of GFLV-F13 RNA1 and RNA2. ORFs are represented by wide open boxes and the VPg is represented by a black circle. The 5′ and 3′ noncoding regions are represented by narrow boxes. Each RNA codes for a polyprotein that is cleaved into functional proteins: VPg, viral genome-linked protein; Pro, proteinase; Pol, RNA-dependent RNA polymerase; HP, homing protein; MP, movement protein; CP, coat protein. Open triangles indicate the cleavage sites.

GFLV is transmitted from grapevine to grapevine by the ectoparasitic nematode *Xiphinema index* that feeds on growing root tips (Raski et al., 1983; Wyss, 2000). Interestingly, *Xiphinema italiae* has also been reported as a vector of GFLV (Cohn et al., 1970); however, this finding has never been confirmed by other investigators (Catalano et al., 1992; Martelli, 1975). Thus, it is unlikely for *X. italiae* to act as a specific vector of GFLV (Brown et al., 1995). Consequently, *X. index* is referred to thereafter in the manuscript as the sole natural vector of GFLV and the transmission process is characterized by a specific and complementary association between *X. index* and GFLV (Brown and Weischer, 1998).

Limited information is available on the molecular determinants involved in the transmission process of GFLV and of nepoviruses in general (McFarlane, 2003). Previous studies indicated that the viral determinants responsible for the specificity of nepovirus transmission map to RNA2. Indeed, pseudorecombinants of Raspberry ringspot virus (RpRSV) and *Tomato black ring virus* (TBRV) showed that transmissibility segregates with RNA2 antigenic properties, suggesting a critical role for protein $2C^{CP}$ in nematode-mediated plant-to-plant spread (Harrison and Murant, 1977; Harrison et al., 1974). Also, protein $2B^{MP}$ has been suspected to play a role in vector specificity based on stretches of identical amino acid sequences among *Xiphinema*-, and also among *Longidorus*-transmitted nepoviruses, and on a lack of identity among amino acid sequences of the two virus groups (Mayo and Robinson, 1996; Mayo et al., 1994).

To investigate the molecular determinants involved in the specific transmission of GFLV by *X. index*, chimeric RNA2 constructs were engineered by replacing the $2A^{HP}$, $2B^{MP}$, or $2C^{CP}$ sequences of GFLV with their counterparts in *Arabis mosaic virus* (ArMV) (Belin et al., 1999), a closely related nepovirus which is transmitted by *Xiphinema diversicaudatum* but not by *X. index* (Brown et al., 1995). Among the recombinant viruses obtained from transcripts of GFLV RNA1 and chimeric RNA2, only those containing the $2B^{MP}$ 9 C-terminal residues and the contiguous $2C^{CP}$ gene (504 aa) of GFLV were transmitted by *X. index* (Belin et al., 2001). To further delineate viral determinants responsible for the specificity of GFLV transmission by *X. index*, we expanded our previous study by examining the potential involvement of the 9 C-terminal residues of protein $2B^{MP}$ and the complete protein $2C^{CP}$. Substitution mutations targeting the four amino acids that are different between GFLV and ArMV in the $2B^{MP}$ C-terminal region were done in single, double, or triple combinations in cDNA clones carrying either the GFLV or the ArMV $2C^{CP}$. We report here that all the hybrid viruses with the $2C^{CP}$ of GFLV are transmitted by *X. index* unlike none with the $2C^{CP}$ of ArMV, regardless of the mutations in the $2B^{MP}$ C-terminal region, demonstrating that protein $2C^{CP}$ is the sole viral determinant for the specific transmission of GFLV by *X. index*.

Results

Engineering of chimeric RNA2 mutants

The viral determinants for the specific transmission of GFLV by *X. index* map to the 513 C-terminal residues of the RNA2-encoded polyprotein, that is, the 9 C-terminal amino acids of protein $2B^{MP}$ and contiguous 504 amino acids of protein $2C^{CP}$ (Belin et al., 2001). To further delineate the viral determinants for specific spread and to clarify the involvement of the $2B^{MP}$ 9 C-terminal residues and the full-length protein $2C^{CP}$, point mutations were introduced into the $2B^{MP}$ C-terminal region in plasmids containing the coat protein (CP) of either GFLV ($pVec2AB_{U9}C$) or ArMV ($pVec2AB_{U9}C_U$) (Belin et al., 2001). These two chimeric plasmids were renamed in this study as $pVec2A_GB_{A9G}C_G$ and $pVec2A_GB_{A9G}C_A$, respectively, with G as subscript indicating the GFLV RNA2 origin of the $2A^{HP}$ and $2C^{CP}$ genes (Serghini et al., 1990), A as subscript indicating the ArMV RNA2 origin of the $2B^{MP}$ and $2C^{CP}$ genes (Loudes et al., 1995), and 9G as subscript indicating the GFLV origin of the 9 C-terminal residues of protein $2B^{MP}$ (Fig. 2A).

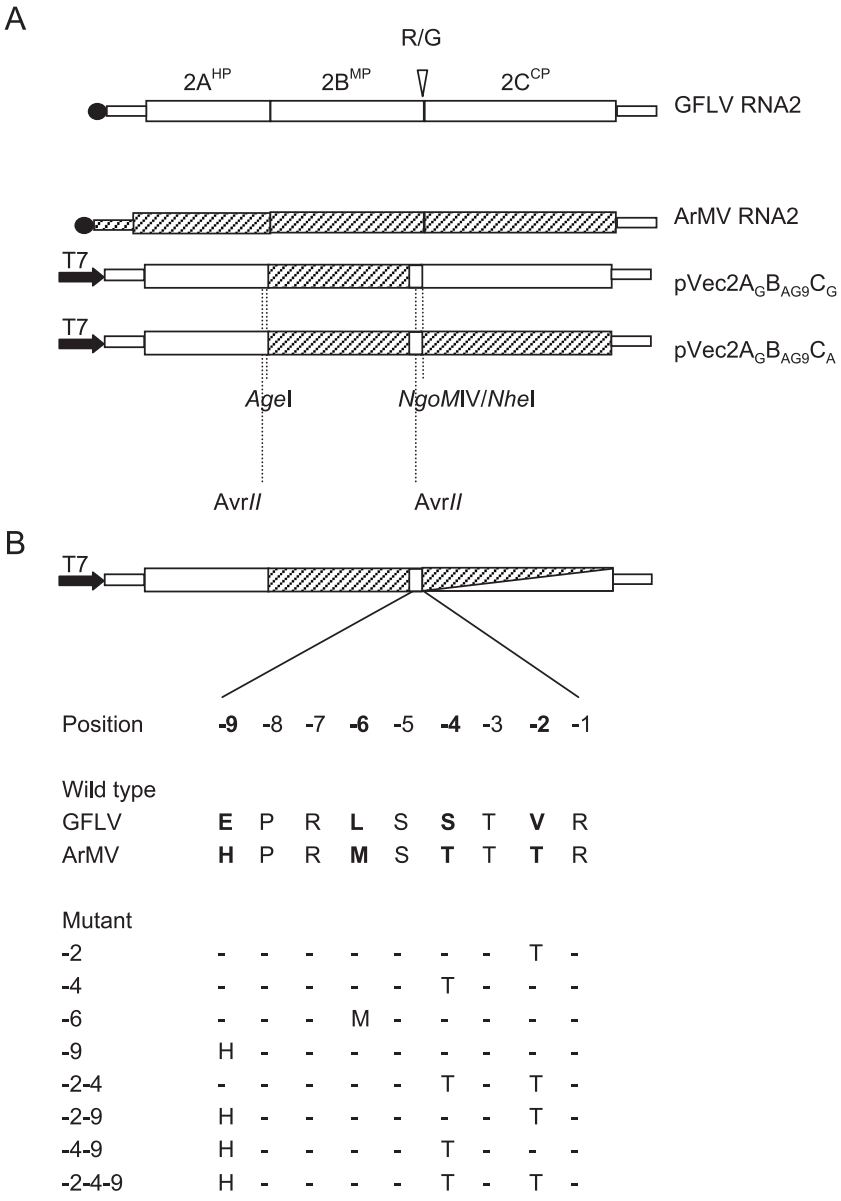

Fig. 2. (A) Schematic representation of wild-type GFLV and ArMV RNA2, and the two chimeric RNA2 constructs used to engineer recombinants. Open boxes indicate GFLV sequences and hatched boxes indicate ArMV RNA2-U sequences. The T7 promoter is represented by a thick black arrow. The position of the *Age*I, *NgoM*IV/*Nhe*I, and *Avr*II restriction sites is indicated by dashed lines. (B) Multiple alignment of the $2B^{MP}$ 9 C-terminal amino acids of wild-type GFLV and ArMV, and each of the eight mutants constructed in $pVec2A_GB_{AG9}C_G$ or $pVec2A_GB_{AG9}C_A$. Amino acid positions are indicated relative to the R/G cleavage site between proteins $2B^{MP}$and $2C^{CP}$. Residues in bold are different between GFLV and ArMV, and "–" denotes conserved residues in all recombinants.

Plasmids $pVec2A_GB_{A9G}C_G$ and $pVec2A_GB_{A9G}C_A$ (Fig. 2A) contain full-length cDNA copies of chimeric GFLV/ArMV RNA2 with the same $2A^{HP}$ and $2B^{MP}$ proteins from GFLV and ArMV origin, respectively, but a different protein $2C^{CP}$. Plasmid $pVec2A_GB_{A9G}C_G$ has the $2C^{CP}$ gene of GFLV, whereas plasmid $pVec2A_GB_{A9G}C_A$ has the $2C^{CP}$ gene of ArMV (Fig. 2A). The transcripts corresponding to $pVec2A_GB_{A9G}C_G$ are functional in planta while those of $pVec2A_GB_{A9G}C_A$ are not (Belin et al., 1999, 2001). In the present study, substitutions were introduced into plasmids: (i) $pVec2A_GB_{A9G}C_A$ to restore the ability of the corresponding transcripts to systemically infect plants in the presence of GFLV RNA1 transcripts (Belin et al., 1999, 2001), and (ii) $pVec2A_GB_{A9G}C_G$ to add as many residues from ArMV origin and yet maintain the infectivity of the corresponding transcripts, respectively.

Of the $2B^{MP}$ 9 C-terminal amino acids, four are different between GFLV and ArMV (Fig. 2B). These four residues are in position 2 (−2), 4 (−4), 6 (−6), and 9 (−9) upstream of the R/G cleavage site between proteins $2B^{MP}$ and $2C^{CP}$. Substitutions targeting these four residues of interest were made in single (−2,−4,−6, and−9), double (−2−4,−2−9 and −4−9), or triple (−2−4−9) combinations by replacing the GFLV amino acids by their counter-

parts in ArMV (Fig. 2B). Amino acid substitutions were introduced in positions −2,−6,−9 because the corresponding residues have different physicochemical properties; residue −2 is hydrophobic non-polar (V) in GFLV and hydrophilic polar (T) in ArMV (Fig. 2B); amino acid −6 is hydrophobic aliphatic (L) in GFLV and hydrophobic with a sulfur group (M) in ArMV (Fig. 2B); amino acid in position −9 is hydrophilic acid (E) in GFLV and hydrophilic basic (H) in ArMV (Fig. 2B). An amino acid substitution was introduced in position −4 because, although hydrophilic in GFLV and ArMV (Fig. 2B), this residue is critical for an efficient cleavage between proteins 2A and $2B^{MP}$ of *Tomato ringspot virus* (ToRSV) (Carrier et al., 1999). This residue is also suspected to play a critical role in the cleavage at the R/G site between proteins $2B^{MP}$ and $2C^{CP}$ of GFLV (Belin et al., 1999).

In total, 16 distinct chimeric RNA2 mutants were engineered (Fig. 2B) by polymerase chain reaction (PCR) using oligonucleotide 38UAge in combination with one of the eight mutagenic oligonucleotides PA00OLI, each carrying either a single, double, or triple mutation (Table 1). The successful insertion of mutagenic PCR fragments in $pVec2A_GB_{A9G}C_G$ and $pVec2A_GB_{A9G}C_A$ was verified by an *Avr*II digestion, releasing a 1081-bp DNA fragment (*Avr*II sites at positions nt 942 and 2023) (Fig. 2A). Engineered RNA2 hybrid molecules will be denoted thereafter in the manuscript by the position of the mutated residue(s) within the 9 C-terminal region of protein $2B^{MP}$. For example, RNA2 recombinant −2 has its GFLV amino acid in position −2, relative to the cleavage site R/G between proteins $2B^{MP}$ and $2C^{CP}$, substituted by its counterpart in ArMV.

Transcripts of the 16 RNA2 mutants (Fig. 2B) were obtained by in vitro transcription with the T7 RNA polymerase (data not shown).

Systemic infection of GFLV RNA1 and chimeric RNA2 mutants in planta

The functionality of the transcripts derived from the 16 chimeric RNA2 mutants was analyzed by mechanical inoculation of *Chenopodium quinoa*, a systemic host, in the presence of GFLV RNA1 transcripts. Our results indicate that 10 of the 16 recombinant viruses (hybrids −2,−4, −6,−9,−4−9 and −2−4−9 with protein $2C^{CP}$ of GFLV, and hybrids −6,−9,−2−9 and −4−9 with protein $2C^{CP}$ of ArMV) were able to systemically infect plants, producing mosaic symptoms in uninoculated apical leaves (Table 2). The presence of virus was confirmed in apical leaves of inoculated plants by double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with immunoglobulins specific to GFLV or ArMV. Interestingly, symptoms appeared 10 days later (21 vs. 7−11 days postinoculation) for hybrid viruses −9 and −2−4−9 with protein $2C^{CP}$ of GFLV in comparison to the other eight functional hybrid viruses. Some of the chimeric viruses (recombinant −2−4−9 with protein $2C^{CP}$ of GFLV, and recombinants −6,−9,−2−9 and −4−9 with protein $2C^{CP}$ of ArMV) also induced chlorotic lesions on inoculated leaves, like the wild-type GFLV strain F13 (Table 2). Furthermore, the 10 functional mutant viruses in *C. quinoa* induced a symptomless systemic infection in *Nicotiana benthamiana*, like the wild-type GFLV strain F13 and wild-type ArMV strain S (data not shown).

Table 1
Oligonucleotides used for the synthesis of mutagenic PCR fragments

Name	Position in		Sequence
	GFLV	ArMV	
PA00OLI2	2106-2157	na	5' TCCTCT**GCCG*GC***TAGCCCTCTCGTGGTTGAGCTCAGCCTAGGCTCTGCGATTCCC 3'
PA00OLI4	2106-2157	na	5' TCCTCT**GCCG*GC***TAGCCCTCTCACGGTAGTGCTCAGCCTAGGCTCTGCGATTCCC 3'
PA00OLI6	2106-2157	na	5' TCCTCT**GCCG*GC***TAGCCCTCTCACGGTTGAGCTCATCCTAGGCTCTGCGATTCCC 3'
PA00OLI9	2111-2157	na	5' TCCTCT**GCCG*GC***TAGCCCTCTCACGGTTGAGCTCAGCCTAGGATGTGCGATTCCCACGTC 3'
PA00OLI24	2106-2157	na	5' TCCTCT**GCCG*GC***TAGCCCTCTCGTGGTAGTGCTCAGCCTAGGCTCTGCGATTCCC 3'
PA00OLI29	2111-2157	na	5' TCCTCT**GCCG*GC***TAGCCCTCTCGTGGTTGAGCTCAGCCTAGGATGTGCGATTCCCACGTC 3'
PA00OLI49	2111-2157	na	5' TCCTCT**GCCG*GC***TAGCCCTCTCACGGTAGTGCTCAGCCTAGGATGTGCGATTCCCACGTC 3'
PA00OLI249	2111-2157	na	5' TCCTCT**GCCG*GC***TAGCCCTCTCGTGGTAGTGCTCAGCCTAGGATGTGCGATTCCCACGTC 3'
38Uage	2111 2157	1093-1121	5' TGCTGTGCTGATGGTAGGACTaccggtGGGC 3'

The restriction sites *NgoM*IV (bold), *Nhe*I (italics), *Age*I (lower case letters), and *Avr*II (underlined) are indicated. The mutagenic nucleotide positions are highlighted in grey.
na: not applicable.

Table 2
Infectivity in *Chenopodium quinoa* plants following mechanical inoculation of GFLV RNA1 transcripts and 16 chimeric RNA2 transcripts mutated in the $2B^{MP}$ 9-C terminal residues

RNA2 mutant[a]	Plasmids from which RNA2 mutants are derived[b]	
	$pVec2A_GB_{A9G}C_G$	$pVec2A_GB_{A9G}C_A$
−2	+[c]	−
−4	+	−
−6	+	+[d]
−9	+	+[d]
−2−4	−	−
−2−9	−	+[d]
−4−9	+	+[d]
−2−4−9	+[d]	−
wild type GFLV	+[d]	na
wild type ArMV	na	+
Mock	−	−

Infectivity of transcripts was evaluated upon mechanical inoculation by monitoring symptom development and assessing the presence of the virus in uninoculated apical leaves by DAS-ELISA using specific γ-globulins to GFLV or ArMV. Positive plants for GFLV had $OD_{405\ nm}$ values of 1.5 ± 0.5 compared to 0.07 ± 0.005 for healthy controls after 90 min of substrate hydrolysis. Positive plants for ArMV had $OD_{405\ nm}$ values of 0.16 ± 0.02 compared to 0.07 ± 0.005 for healthy controls after 90 min of substrate hydrolysis. For each mutant, 2–8 plants were inoculated in four independent experiments.
na: not applicable.
[a] See Fig. 2B for a description of the mutated amino acids in the $2B^{MP}$ C-terminal region.
[b] See Fig. 2A for a description of the parental plasmids $pVec2A_GB_{A9G}C_G$ and $pVec2A_GB_{A9G}C_A$.
[c] Occurrence (+) or lack (−) of systemic plant infection.
[d] Chlorotic lesions on inoculated leaves.

The RNA2 progeny of recombinant viruses derived from transcripts of GFLV RNA1 and mutant RNA2 was characterized in infected *C. quinoa* by immunocapture-reverse transcription-PCR (IC-RT-PCR) and sequencing (Fig. 3A) using six distinct sets of primers (Table 4). Sequence data showed a stable inheritance of all the mutations within the $2B^{MP}$ C-terminal region upon infection in planta. In addition, no compensatory mutation was observed in protein $2C^{CP}$ for any of the recombinants. Only one silent mutation (T^{2625} for C^{2625}) was identified in the progeny of all the hybrid viruses with the $2C^{CP}$ gene of GFLV.

Transmissibility of recombinant viruses by X. index

The transmissibility of chimeric viruses by *X. index* was tested in the greenhouse with aviruliferous nematode vectors reared on fig plants, a host for nematodes but not for GFLV. *N. benthamiana* seedlings were mechanically inoculated with one of each of the 10 functional hybrid viruses by using crude sap of *C. quinoa* infected with GFLV RNA1 and chimeric RNA2 transcripts as inoculum. Infected *N. benthamiana* was further used as virus source plants in nematode transmission tests.

A two-step transmission procedure was used, comprising a virus acquisition step followed by a virus transmission step. The first step consisted of an exposure of aviruliferous nematodes to roots of infected *N. benthamiana* plants for 6 weeks. The second step consisted of uprooting the *N. benthamiana* virus source plant, replacing it with a healthy rooted grapevine cutting, and allowing the nematodes to feed for an additional 6 weeks. Remarkably, among the 10 functional hybrid viruses, the six with protein $2C^{CP}$ of GFLV (−2,−4,−6,−9,−4−9, and −2−4−9), but none of the four with protein $2C^{CP}$ of ArMV (−6,−9,−2−9, and −4−9), were readily transmitted by *X. index*, as shown by DAS-ELISA (Table 3).

The recombinant viruses with protein $2C^{CP}$ of GFLV induced a systemic infection of the recipient grapevine

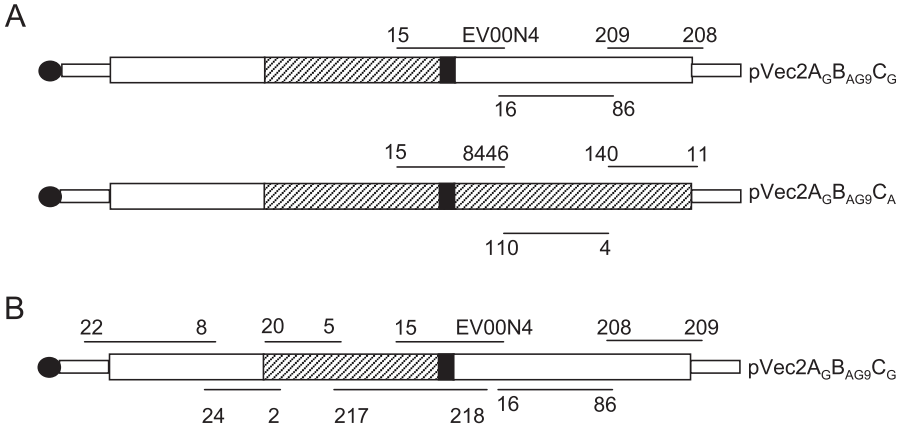

Fig. 3. Strategy to sequence RNA2 progeny of mutant viruses after (A) systemic infection and (B) nematode transmission. The primers used for IC-RT-PCR are indicated at the extremities of the corresponding amplified cDNA fragments. GFLV and ArMV sequences are represented as open or hatched boxes, respectively, and the C-terminal region of protein $2B^{MP}$ is indicated by a black box. The 5′and 3′noncoding regions are denoted by narrow boxes and the VPg is represented by a black circle. The sequence and location of each primer are given in Table 4.

Table 3
Transmissibility of natural wild-type GFLV-F13 and ArMV-S, and mutant viruses by *X. index*

Virus	Mutant[a]	Transmission[b]	%
GFLV	−2	10/12	83
	−4	7/12	53
	−6	12/12	100
	−9	7/12	53
	−4−9	10/15	66
	−2−4−9	13/15	86
	wild-type	14/14	100
ArMV	−6	0/18	0
	−9	0/7	0
	−2−9	0/15	0
	−4−9	0/13	0
	wild type	0/17	0
Mock		0/3	0

Positive GFLV-recipient plants had $OD_{405\ nm}$ values of 0.8 ± 0.5 compared to 0.025 ± 0.015 for healthy controls after 90 min of substrate hydrolysis with specific γ-globulins to GFLV. Negative ArMV recipient plants had $OD_{405\ nm}$ values of 0.025 ± 0.005 compared to 0.025 ± 0.005 and 1.2 ± 0.2 for healthy and infected controls, respectively, after 90 min of substrate hydrolysis with specific γ-globulins to ArMV. Each bait recipient plant was maintained for 6 weeks in contact with *X. index* which previously fed on a single *N. benthamiana* virus source.

[a] See Fig. 2B for a description of the hybrid viruses.

[b] Data represent the number of recipient plants that reacted positively for GFLV or ArMV in ELISA over the total number of bait plants tested.

plants, demonstrating their functionality upon *X. index*-mediated transmission.

Characterization of the RNA2 progeny of X. index-transmitted recombinant viruses

The coding region of the RNA2 progeny of the six recombinant viruses that were transmitted by *X. index* was characterized by IC-RT-PCR and sequencing (Fig. 3B) using seven pairs of primers (Table 4) and crude root extracts from recipient plants. Sequence data showed the stability of the progeny although a limited number of nucleotide changes was identified in the $2A^{HP}$, $2B^{MP}$, and $2C^{CP}$ regions of some of the recombinant viruses. Indeed, only one or two of the 3310–3380 (0.03–0.06%) RNA2 nucleotides were modified compared to the reference GFLV (Serghini et al., 1990) and ArMV (Loudes et al., 1995) sequences. All the nucleotide modifications were also found in the virus source plant corresponding to the recipient plant from which the sequenced fragment was obtained.

In the $2C^{CP}$ gene, recombinant virus −2 with the $2C^{CP}$ of GFLV had nucleotide C^{2610} changed into T^{2610}. This modification is silent. Also, hybrid −2−4−9 with the $2C^{CP}$ of GFLV had nucleotide A^{3490} substituted for G^{3490}, altering amino acid K^{1164} into E^{1164}. These modifications are likely nonessential for virus transmission because they were not identified in the RNA2 progeny of all the recombinant viruses. Our previous findings indicated that proteins $2A^{HP}$ and $2B^{MP}$, except its 9 C-terminal residues, are not responsible for the specificity of GFLV transmission (Belin et al., 2001). Nonetheless, we sequenced the $2A^{HP}$ and $2B^{MP}$ genes to assess potential compensatory mutations. In the $2A^{HP}$ gene, hybrid −2−4−9 with the $2C^{CP}$ of GFLV had nucleotide G^{718} substituted for A^{718}, altering amino acid G^{240} into S^{240}. In the $2B^{MP}$ gene, recombinant virus −6 with the $2C^{CP}$ of GFLV had nucleotide A^{2031} changed into T^{2031}; this mutation converted E^{677} into D^{677}. The fact that these limited modifications were found in the progeny of only two of the six *X. index*-transmitted viruses indicates that compensatory changes did not emerge upon transmission.

Additional nucleotide changes were also detected compared to the reference GFLV (Serghini et al., 1990) and

Table 4
Oligonucleotides used for sequencing the RNA2 progeny of recombinant viruses

Name	GFLV position	ArMV position	Sequence
22	138–158		5′ CTTTTTTGTTCTTTTATTTTGCGC 3′
8	851–869		5′ GAGGATTTTGGATTGGGGG 3′
20		1095–1125	5′ TGCTGTGCTGATGGTAGGACTACCGGTGGGC 3′
5		1560–1579	5′ TTGCCTGGCATTCCAAAAGG 3′
24	784–803		5′ GTGGTATGACACTAGTGATG 3′
2		1254–1273	5′ TCAGTTTGAGCAGCAAGACC 3′
217		1443–1464	5′ ACTGGGGAGAATACTTTAGAAA 3′
218	2331–2353		5′ GCCCAACTTATGGTGGATAAGCC 3′
15		1714–1731	5′ AAGAAGAACCGAGGCGCG 3′
8446		2344–2326	5′ CTCATAACCCACGTAATAC 3′
EV00N4	2404–2385		5′ CCACATDGCRTGACCACACA 3′
86	2970–3000		5′ CTGAAGATATCGATAGGTCCTGCTATTGCATAAA 3′
16	2234–2254		5′ GGATTGACATGGGTGATGAGC 3′
208	3700–3719		5′ AAGTGTGTCCAAAGGACAAA 3′
209	2762–2781		5′ ATTTGTGCGCCAATCTTCTA 3′
4		2931–2962	5′ TCCTCGATGTAACACCCGGGGTATTTAAACAG 3′
110		2254–2280	5′ AGTGGATTACTGCAGGACTTGTTATGC 3′
11		3570–3590	5′ ACACTTGGGTCTTTTAAAGTC 3′
140		2858–2875	5′ TAGCCCTTGCACTTATGG 3′

R = A or G.
D = A or G or T.

ArMV (Loudes et al., 1995) sequences. These modifications probably originated during the engineering process of the recombinant viruses because they were found in the progeny of all the recombinant viruses after nematode transmission and in plasmid pVec2$A_GB_{A9G}C_G$ as well. In the $2A^{HP}$ gene, residue T^{306} was changed into A^{306} and an additional Q^{165} was identified. In the $2B^{MP}$ gene, amino acids A^{593}, V^{364}, and R^{370} were changed into P^{593}, R^{364}, and K^{370}. More unexpectedly, a deletion of three amino acids (^{365}CCA^{367}) was found at the N-terminus of protein $2B^{MP}$. For recombinant virus −9 with the $2C^{CP}$ of GFLV, $2B^{MP}$ amino acid A^{618} was substituted for V^{618}. In the $2C^{CP}$ gene of ArMV origin, residue S^{1100} was converted into A^{1100}. Remarkably, these deletions did neither affect the transmissibility of recombinant viruses nor cell-to-cell and long distance movement. Taken together, our sequence information demonstrated that recombinant viruses with the $2C^{CP}$ of GFLV are transmitted by *X. index* with minor and nonessential modifications.

Discussion

We engineered 16 mutants from GFLV/ArMV chimeric RNA2 to clarify the involvement of $2B^{MP}$ 9 C-terminal residues and protein $2C^{CP}$ in the specific transmission of GFLV by *X. index*. Transmissibility assays clearly showed that, regardless of the mutations within the $2B^{MP}$ C-terminal region, all the recombinant viruses with protein $2C^{CP}$ of GFLV were transmitted by *X. index* unlike none with protein $2C^{CP}$ of ArMV. Because earlier experiments demonstrated that the N-terminal 340 residues of protein $2B^{MP}$ are not responsible for the specificity of transmission (Belin et al., 2001), we can now definitely exclude the complete protein $2B^{MP}$ from the specific spread of GFLV by *X. index*. Consequently, because protein $2A^{HP}$ was also excluded previously (Belin et al., 2001), we can conclude that protein $2C^{CP}$ is the sole viral determinant for the specific transmission of GFLV by *X. index*. This is the first direct molecular evidence illustrating the involvement and critical role of protein $2C^{CP}$ in the transmission specificity of a nepovirus.

The engineering of viable and systemically infectious recombinant viruses with the CP of either GFLV or ArMV was a major breakthrough in addressing the molecular basis of a nepovirus transmission. This technical achievement enabled us to overcome some of our previous difficulties associated to nonfunctional constructs (Belin et al., 1999, 2001) and further identify the viral determinants involved in the specific association between GFLV and *X. index*.

Earlier observations strongly suggested the implication of protein $2C^{CP}$ in the transmission process of GFLV and other nepoviruses. Electron microscopy studies revealed monolayers of GFLV particles adhering to the cuticular lining of the oesophageal tract in the anterior part of the odontophore of *X. index* (Taylor and Robertson, 1970). Similar observations were reported for ArMV in *X. diversicaudatum* (Taylor and Robertson, 1970). Also, the *Longidorus elongatus*-mediated transmission of pseudorecombinant isolates of TBRV correlated with the antigenic properties of virus particles (Harrison and Murant, 1977). However, these studies did not provide any information on the implication of the coat protein (CP) in the specificity of the transmission process. Our work demonstrates that nepoviruses require only the CP for vector specificity, assuming other species of this plant virus genus share identical features to GFLV. It would have been valuable to confirm our findings by investigating the transmissibility of recombinant viruses with the nematode vector of ArMV. In particular, it would have been interesting to examine whether hybrid viruses with protein $2C^{CP}$ of ArMV are transmitted by *X. diversicaudatum* and those with protein $2C^{CP}$ of GFLV are not. Unfortunately, active *X. diversicaudatum* rearings were not available to us throughout the course of our study to carry out such experiments.

Virus species from different plant virus genera seem to have developed unique strategies for their vector-assisted transmission. In the case of tobravirus species, the CP and the nonstructural protein 2b are essential for nematode transmission, and protein 2c enhances transmissibility (Brown and MacFarlane, 2001; McFarlane, 2003). In the case of aphid-transmitted viruses, several viral proteins are involved in the transmission process. The CP is the only viral-encoded protein involved in the transmission of *Cucumber mosaic virus* (CMV) from the genus *Cucumovirus* in the family *Bromoviridae* (Chen and Francki, 1990). Interestingly, distinct CP motifs are responsible for the transmission of CMV by different aphid species (Perry et al., 1998). For potyviruses in the family *Potyviridae*, the CP and a helper component are required for aphid transmission (Pirone and Blanc, 1996). For *Cauliflower mosaic virus* from the genus *Caulimovirus* in the family *Caulimoviridae*, the CP, protein P2, which is an aphid transmission factor, and protein P3, which bridges protein P2 and virus particles, are necessary (Drucker et al., 2002). The CP is also essential for transmission and vector specificity of gemini- (Hönle et al., 2001) and tombusviruses (McLean et al., 1994). Because our study primarily addressed the specificity of transmission, we cannot rule out the involvement of one or more other GFLV-encoded protein(s), in addition to protein $2C^{CP}$, in the transmission process.

Of the recombinant viruses with the $2C^{CP}$ of GFLV, none with a single mutation in the $2B^{MP}$ C-terminal region abolished their infectivity but hybrid viruses −2−4 and −2−9 with a double mutation were not infectious in planta. For recombinant viruses with the $2C^{CP}$ of ArMV, mutations affecting amino acid −6,−9,−4−9 and −2−9 restored the functionality virus movement of the initially noninfective chimeric transcripts derived from plasmid pVec2$A_GB_{A9G}C_A$. Also, amino acid substitutions in positions −6 and −9 did not affect

systemic infection regardless of the CP source, while recombinants −2−4−9 and −2−9 only functioned with the CP of GFLV and ArMV, respectively. Altogether, these results indicate no strict symmetrical correlation between mutations and infectivity in planta of transcripts derived from constructs pVec2$A_G B_{A9G} C_G$ and pVec2A_G-$B_{A9G} C_A$ (Table 2), suggesting the functional importance of secondary structures or the charge of the $2B^{MP}$ 9 C-terminal residues. These molecular features are likely critical for an effective interaction between proteins $2B^{MP}$ and $2C^{CP}$ for virus spread. Consistent with this hypothesis, we recently found a lack of correlation between a systemic plant infection and a complete processing at the R/G cleavage site between proteins $2B^{MP}$ and $2C^{CP}$ of the 10 functional RNA2 chimeric constructs developed in this study (Andret-Link et al., unpublished results).

GFLV like CPMV spreads from cell to cell as virions through tubular structures constituted by the viral-encoded movement protein (MP) (Ritzenthaler et al., 1995). In the case of CPMV, the N-terminal and central regions of the MP are involved in tubule formation and the C-terminal domain probably interacts with virus particles (Bertens et al., 2003). In vitro serological assays further showed that the MP C-terminal 48 residues constitute the specific virion-binding domain (Carvalho et al., 2003). In the case of GFLV, limited information is available on the interaction between MP and CP for virus spread, although it has been suggested that the 9 C-terminal residues of $2B^{MP}$ should be of the same virus origin as the $2C^{CP}$ for systemic virus spread (Belin et al., 1999). Interestingly, our study highlighted the critical role of the structure or the charge rather than the sequence of the 9 C-terminal residues of GFLV $2B^{MP}$ for systemic infection eventually through an effective interaction with protein $2C^{CP}$. Based on these data, it would be valuable to determine the crystal structure of protein $2B^{MP}$ to visualize the configuration of its C-terminal region and further investigate the specific $2B^{MP}$–$2C^{CP}$ interaction.

X. index-transmitted recombinant viruses were stable because their RNA2 progeny carried only limited and minor alterations in the encoded polyprotein. In addition, the nucleotide and amino acid modifications found affected individual but not all the recombinant viruses. Because amino acids essential for virus spread are expected to be present in the RNA2 progeny of all nematode-transmitted hybrids, the alterations probably reflect permissible variations in the viral genome.

Our previous *X. index*-mediated transmission experiments were carried out with infected rooted grapevine cuttings as source plants. Because mechanical inoculation of grape plants is difficult, GFLV-infected cuttings were obtained through in vitro heterologous grafting (Belin et al., 2001). This approach is efficient but very tedious and time consuming. Here, we used *N. benthamiana* as virus donor in transmission assays. The use of this non-host plant of *X. index* substantially reduced the time needed to prepare the plant material and test the nematode transmissibility of the hybrid viruses from 24 to 12 weeks. Nonetheless, all the 10 recombinant viruses that systemically infect *C. quinoa* and *N. benthamiana* were successfully transmitted to grapevine plants by in vitro heterologous grafting with ELISA $OD_{405\ nm}$ values of grapevines root or leaf extracts similar to those observed in infected herbaceous hosts.

To further identify the $2C^{CP}$ residues for transmission specificity, we hypothesized that potential determinants are likely located on the external surface of the virus capsid in order for virions to interact with potential receptors at specific retention sites in the food canal of nematodes. Thus, a 3D model of the GFLV capsid was recently constructed using the crystal structure of ToRSV, the type member of the genus *Nepovirus* (Chandrasekar and Johnson, 1997). The predicted surface topography of the GFLV capsid was examined to identify the amino acids specific to GFLV and conserved among numerous GFLV isolates (Andret-Link et al., unpublished results). Substitution experiments targeting these residues are in progress to validate our model and further investigate the functionality of the $2C^{CP}$ amino acids of interest.

Materials and methods

Virus strains and plants

GFLV strain F13 (Vuittenez et al., 1964) and ArMV strain S (Huss et al., 1989) were isolated from naturally infected grapevines and subsequently propagated in *Chenopodium quinoa*, a systemic host for both viruses. Genomic RNAs extracted from purified preparations of these two virus strains were used to synthesize full-length cDNA clones of GFLV RNA1 and RNA2 (Viry et al., 1993) and ArMV RNA2-U (Loudes et al., 1995), respectively. GFLV strain F13 and ArMV strain S were also used as positive and negative controls in nematode transmission tests.

Chenopodium quinoa plants were used to test the infectivity of transcripts. *N. benthamiana* and the grapevine rootstock Kober 5BB (*Vitis berlandieri* × *V. riparia*) were used in nematode transmission experiments as virus donor and virus recipient plants, respectively.

Point mutation experiments affecting chimeric cDNA of RNA2

Plasmid pVec is a pUC-derived vector containing the 5′ and 3′ noncoding regions of GFLV RNA2 under the control of promoter T7. Plasmid pVec was used to produce plasmids pVec2ABC, pVec2$AB_{U9}C$, and pVec2$AB_{U9}C_U$ (Belin et al., 1999). These plasmids contain a full-length cDNA copy of either GFLV RNA2 (pVec2ABC) or chimeric RNA2 with protein $2A^{HP}$ of GFLV, protein $2B^{MP}$ of ArMV, except for its 9 C-terminal amino acids which are from GFLV origin,

and protein $2C^{CP}$ of GFLV ($pVec2AB_{U9}C$) or ArMV ($pVec2AB_{U9}C_U$). Of the two biologically indistinguishable RNA2 constituents of ArMV strain S, called RNA2-U and RNA2-L (Loudes et al., 1995), only ArMV RNA2-U was used in this study. The chimeric plasmids $pVec2AB_{U9}C$ and $pVec2AB_{U9}C_U$ were renamed in this study as $pVec2A_GB_{A9G}C_G$ and $pVec2A_GB_{A9G}C_A$, respectively.

Point substitutions affecting the $2B^{MP}$ 9 C-terminal residues were introduced by cloning PCR fragments carrying the mutations into plasmids $pVec2A_GB_{A9G}C_G$ and $pVec2A_GB_{A9G}C_A$. PCR fragments carrying mutations were synthesized using appropriate primers selected among eight mutagenic oligonucleotides and plasmid $pVec2A_GB_{A9G}C_G$ as template. Each pair of primers was composed of oligonucleotides PA00OLI and 38Uage (Table 1). Oligonucleotide primers PA00OLI had one of the mutations, *Nhe*I and *NgoM*IV restriction sites, and a silent mutation introducing an additional *Avr*II restriction site at position nt 2023 (this *Avr*II site was used to check the successful insertion of the mutated DNA fragments into plasmids $pVec2A_GB_{A9G}C_G$ and $pVec2A_GB_{A9G}C_A$). The second oligonucleotide 38UAge contains an *Age*I restriction site (Table 1). Each PCR reaction was carried out in a 50 μl final volume with 10 ng of vector, 0.2 mM of each dNTP, 1.25 U *Taq* DNA polymerase, 1.5 mM $MgCl_2$, 50 pmol of each primer, 50 mM KCl, 10 mM Tris–HCl pH 9, and 0.1% Triton X-100. The PCR included a 2 min denaturation step at 94 °C followed by 30 cycles of 30 s melting at 94 °C, 1 min annealing at 60 °C, and 2 min elongation at 72 °C with a final extension of 7 min at 72 °C. The PCR fragments were purified by electrophoresis on a 1% low melting point agarose gel, digested by *Age*I and *Nhe*I or *Age*I and *NgoM*IV, and cloned into $pVec2A_GB_{A9G}C_G$ digested with *Nhe*I and *Age*I, and $pVec2A_GB_{A9G}C_A$ digested with *NgoM*IV and *Age*I, respectively.

In vitro transcription

Plasmid pMV13 was linearized with *Bgl*II to produce transcripts of GFLV RNA1 (Tr1) (Viry et al., 1993). Plasmids pVec2ABC, $pVec2A_GB_{A9G}C_G$, $pVec2A_GB_{A9G}C_A$, and $pVec2A_GB_{A9GX}C_A$ (x as subscript indicates mutagenesis of some of the $2B^{MP}$ 9 C-terminal residues) were linearized with *Sal*I to produce wild-type, chimeric, and mutated chimeric transcripts of RNA2, respectively. Capped transcripts were synthesized in vitro using the RiboMAX system according to the manufacturer's instructions (Promega). The size and integrity of the transcripts were checked by agarose-formaldehyde gel electrophoresis.

Mechanical inoculation of C. quinoa and N. benthamiana

C. quinoa plants were mechanically inoculated according to Viry et al. (1993) with purified wild-type virus (GFLV strain F13 and ArMV strain S) or with mutated RNA2 transcripts associated with Tr1. Seedlings of *C. quinoa* were mechanically inoculated at the 4–6 leaf development stage with 5 and 10 μg of RNA1 and mutated RNA2 transcripts by plant, respectively. Crude sap from infected *C. quinoa* leaves was used as inoculum to infect *N. benthamiana* plants by rubbing carborundum-dusted leaves.

Nematode transmission assays

The nematode transmissibility tests were performed under greenhouse conditions using aviluriferous *X. index* isolated from rearing on fig plants (*Ficus carica*). Nematodes were extracted from soil samples using the sieving method described by Flegg (1967) and subsequently counted with a binocular microscope. A two-step transmission procedure was used. The first step consisted of feeding ca. 300 nematodes for 6 weeks on a single virus source plant in 0.5 l plastic pots containing two-thirds of sand and one-third of loess. Infected *N. benthamiana* was used as virus source plants. Because GFLV induces a symptomless infection in *N. benthamiana*, the presence of the virus was verified by DAS-ELISA in the leaves and young roots of each source plant at the end of the acquisition step. After the acquisition step, the virus source plant was removed from the pots and replaced by a healthy recipient grapevine. The nematode feeding period lasted for six more weeks. After this period, the grapevines were uprooted and nematode transmissibility was assessed in rootlets of the recipient grapevines by serological assays. Leaves of the recipient grapevine plants that became infected after nematode transmission were analyzed by DAS-ELISA to verify the long distance movement of viruses from roots to the leaves. For each transmission test, the wild-type GFLV strain F13 and ArMV strain S were used as positive and negative control, respectively.

Characterization of recombinant RNA2 progeny

The progeny of mutated RNA2 was characterized by IC-RT-PCR in infected plants after *X. index*-mediated transmission. Crude sap of roots of recipient plants was incubated overnight at 4 °C in microtubes coated with specific GFLV-F13 or ArMV-S immunoglobulins. Tubes were washed three times with phosphate-buffered saline (150 mM NaCl, 1.5 mM KH_2PO_4, 10 mM Na_2HPO_4, 2 mM KCl) containing 0.05% Tween 20 at room temperature. Virus particles were disrupted and viral RNAs released by heating 10 min at 65 °C in 10 μl of sterile water. Reverse transcription was performed with the RNA-dependent DNA polymerase of *Avian myeloblastosis virus* according to the manufacturer's instructions (Promega) and cDNA products were amplified by PCR using the Taq DNA polymerase (Promega) and appropriate primers (Fig. 3). PCR-amplified fragments were purified with the QIAquick gel extraction kit (Qiagen) and sequenced using the ABI Prism Big Dye Terminator kit and

an Applied Biosystems 3100 sequencer at the Sequencing Facility of the Institut de Biologie Moléculaire des Plantes, Centre National de la Recherche Scientifique, in Strasbourg, France.

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